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(54) Title: A METHOD OF PLANT TRANSFORMATION

(57) Abstract: The present invention relates generally to a method for transforming plant cells with genetic material and to plants and plantlets regenerated from transformed plant cells. The present invention further relates to progeny and reproductive material, including pollen, seeds and propagating tissue, from plants and plantlets regenerated from transformed plant cells. The present invention further provides callus, explant and other plant tissue from regenerated plants. The present invention provides the means to genetically modify plants and in particular monocotyledonous plants and even more particularly pineapple plants and related species.

A METHOD OF PLANT TRANSFORMATION

FIELD OF THE INVENTION

The present invention relates generally to a method for transforming plant cells with genetic material and to plants and plantlets regenerated from transformed plant cells. The present invention further relates to progeny and reproductive material, including pollen, seeds and propagating tissue, from plants and plantlets regenerated from transformed plant cells. The present invention further provides callus, explant and other plant tissue from regenerated plants. The present invention provides the means to genetically modify plants and in particular monocotyledonous plants and even more particularly pineapple plants and related species.

BACKGROUND OF THE INVENTION

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other country.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

The genetic improvement of food crops and ornamental plants requires efficient methods of introducing genetic material into plant cells and the ability to regenerate plants therefrom. A range of methods has been developed to transform plant cells with genetic material. These methods include protoplast transformation procedures, microparticle bombardment and *Agrobacterium*-mediated transformation. The latter method is a well known method for transforming cells of dicotyledonous plants but has been less widely adaptable to monocotyledonous plants.

Pineapple (*Ananas spp*) is an agronomically important food crop for the canned and processed food industries as well as the fresh fruit industries. Genetic manipulation of many of the important cultivars of pineapple has been difficult. Conventional breeding practices have been particularly difficult due to the self-incompatibility of pineapple and the lengthy period between successive fruit generations.

International Patent Application No. PCT/US98/03681 [WO98/36637] purports to provide a means of transforming pineapple embryogenic callus with DNA. However, the establishment of embryogenic callus can be time consuming, is labour intensive and is not always successful. There is a need, therefore, to establish more efficacious procedures to transform pineapple cells and cells of other monocotyledonous plants with genetic material. This will then enable the rapid genetic manipulation of monocotyledonous plants to introduce desirable traits or to remove less desirable traits.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier, i.e. <400>1, <400>2, etc. A sequence listing is provided after the claims.

The present invention provides a method for transforming cells of monocotyledonous plants and more particularly plants of the Bromiliadeae family such as pineapples or ornamental plants as well as plants outside the Bromiliadeae family such as but not limited to bananas, ginger, sugar cane and cereal plants. The present invention further provides plants regenerated following the transformation procedure and in particular plants which are genetically modified such as to express a useful trait. The transformation process involves co-cultivating plant explant with a species of *Agrobacterium* and then culturing the potentially transformed cells under selection conditions including conditions sufficient to initiate transformation of organogenic callus to permit the selection of transformed cells. Plants are then regenerated from the selected callus.

Accordingly, one aspect of the present invention contemplates method of transforming cells of a monocotyledonous plant with genetic material, said method comprising:-

obtaining an explant from said plant;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without said *Agrobacterium* overgrowing the plant cells; and

selecting for the transformed plant cells and permitting the cells to form organogenic callus.

Another aspect of the present invention provides a method for producing a genetically modified monocotyledonous plant, said method comprising:-

obtaining explant from a plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transformed into said plant cells for a time and under conditions sufficient for the genetic material to transfer to plant cells without said *Agrobacterium* overgrowing the plant cells;

selecting transformed plant cells and permitting the cells to form organogenic callus; and

regenerating a plant from selected transformed plant cells.

A further aspect of the present invention provides a method for producing a genetically modified monocotyledonous plant, said method comprising:

obtaining an explant from said plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without the *Agrobacterium* overgrowing the plant cells;

selecting for the transformed plant cells and permitting the cells to form organogenic callus; and

regenerating a plant from said transformed organogenic callus.

Still another aspect of the present invention provides a method of transforming cells of a pineapple plant or a related plant with genetic material, said method comprising:-

obtaining explant from said pineapple plant or a related species;

co-cultivating same with *Agrobacterium* species having T-DNA or T-DNA region comprising genetic material to be transformed into said pineapple plant cells for a time and under conditions sufficient for transfer of the genetic material to occur; and

selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus.

In a related embodiment, the present invention contemplates a method of genetically modifying a pineapple or related plant, said method comprising:-

obtaining an explant from a pineapple or related plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transferred into the pineapple or related cells for a time and under conditions sufficient for the genetic material to transfer to said cells;

selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus; and

regenerating a pineapple or related plant from said organogenic callus.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation of the binary vector, pART27, and representations of the derivatives pART27.GUS,pBS427.GUS and pBS427.A map of pART27 is shown at the bottom; the thin line represents the plasmid backbone; functional segments of DNA associated with selection of transgenic plants are represented diagrammatically by boxes and arrows. For pART27, these are, from left to right: the black arrow, which represents the right border (RB) responsible for T-DNA transfer; the adjacent arrow, which represents the *lacZ* gene, it also contains a unique *Not*I restriction site; adjacent to this is a selection cassette consisting of the nos promoter, the *npt*II gene and a nos 3' terminator (the small black arrow above the nos promoter indicates the transcription start site); the left border (LB) for T-DNA transfer lies adjacent to this. For the binary vector, pBS427, the Sc4-*npt*II (intron)-Sc5 3' selection cassette is shown in the diagram above that of pART27. For pBS427, this selection cassette replaces that of pART27 as indicated by the lines. In the pBS427 selection cassette, the Sc4 promoter drives *npt*II expression, the *npt*II gene in this construct contains an intron, indicated by the black box, transcription of this gene stops at an Sc5 3' terminator. The GUS expression cassette from pART7.GUS, shown at the top left of the diagram, was cloned into the *Not*I site of pART27, as indicated by the lines. For pBS427.GUS, pART7.GUS was cloned into the equivalent *Not*I site of pBS427.

Figure 2 is a photographic representation showing non-transformed (GUS negative) tissue (shown on the left). Next to this are three pieces of GUS-expressing callus which stain blue; shown next to this are three transgenic shoots which also stain blue.

Figure 3 is a photographic representation of brightly fluorescent callus visualized approximately three months following co-cultivation with pBIN.mGFP5-ER. Fluorescence is very bright; non-transformed, GFP negative callus, is barely visible below this.

Figure 4 is a photographic representation of agarose gel electrophoresis showing PCR reactions confirming the presence of the *npt*II gene in transgenic pineapple shoots. Appropriate controls and DNAs prepared from a total of six transgenic shoots were analysed by PCR using the "*npt*II oligos"; see <400>1 and <400>2. Transformed shoot DNAs were derived from two separate transformation events using pBIN.mGFP5-ER, three shoots from each event. Lanes are 1: MW Marker (Marker III Boehringer Manheim); 2: No-DNA control; 3: non-transgenic pineapple DNA control; 4: Known transgenic pineapple control DNA; 5-7: DNAs from 3 shoots derived from pBIN.mGFP5-ER-transformed shoots (callus no 1A); 8-10: DNAs from 3 shoots derived from pBIN.mGFP5-ER-transformed shoots (callus no 2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a method of transforming monocotyledonous cells and to a method for regenerating genetically modified plants therefrom.

Accordingly, one aspect of the present invention contemplates method of transforming cells of a monocotyledonous plant with genetic material, said method comprising:-

obtaining an explant from said plant;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without said *Agrobacterium* overgrowing the plant cells; and

selecting for the transformed plant cells and permitting the cells to form organogenic callus.

In a related embodiment, the present invention provides a method for producing a genetically modified monocotyledonous plant, said method comprising:-

obtaining explant from a plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transformed into said plant cells for a time and under conditions sufficient for the genetic material to transfer to plant cells without said *Agrobacterium* overgrowing the plant cells;

selecting transformed plant cells and permitting the cells to form organogenic callus; and

regenerating a plant from selected transformed plant cells.

One skilled in the art will appreciate that the order of the various steps of the transformation process may vary or alter and the separation of the steps into discrete paragraphs above is not to be construed that the order of method steps cannot be altered.

The term "genetically modified" is used in its broadest sense to include introducing exogenous genetic material such as in the generation of a transgenic plant as well as inducing a mutation in the genome of a plant cell. The term also encompasses introducing antisense molecules, ribozymes and sense molecules such as for use in co-suppression. A "mutation" includes the introduction of a single or multiple nucleotide substitution, addition and/or deletion. The genetic material to be introduced may be a gene or may correspond to a gene or may be a gene fragment, segment, portion and/or a gene hybrid or fusion or a combination of genes. The genes may be in monocistronic form or in multicistronic form.

The term "gene" is used in its broadest sense and includes cDNA corresponding to the exons of a gene. Accordingly, reference herein to a "gene" is to be taken to include:-

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product.

The genetic material may be in the form of a genetic construct comprising a gene or nucleic acid molecule to be introduced into a plant cell, operably linked to a promoter and optionally various regulatory sequences.

The genetic material of the present invention may comprise a sequence of nucleotides or be complementary to a sequence of nucleotides which comprise one or more of the following: a promoter sequence, a 5' non-coding region, a *cis*-regulatory region such as a functional binding site for transcriptional regulatory protein or translational regulatory protein, an upstream activator sequence, an enhancer element, a silencer element, a TATA box motif, a CCAAT box motif, or an upstream open reading frame, transcriptional start site, translational start site, and/or nucleotide sequence which encodes a leader sequence.

The term "5' non-coding region" is used herein in its broadest context to include all nucleotide sequences which are derived from the upstream region of an expressible gene, other than those sequences which encode amino acid residues which comprise the polypeptide product of said gene, wherein the 5' non-coding region confers or activates or otherwise facilitates, at least in part, expression of the gene.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or environmental stimuli, or in a tissue-specific or cell-type-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. The present invention extends to any promoter or promoter element recognized by one or more of a type I, II and/or III DNA polymerase.

In the present context, the term "promoter" is also used to describe a synthetic or fusion

molecule, or derivative which confers, activates or enhances expression of a structural gene or other nucleic acid molecule, in a plant cell. Preferred promoters according to the invention may contain additional copies of one or more specific regulatory elements to further enhance expression in a cell, and/or to alter the timing of expression of a structural gene to which it is operably connected.

The term "operably connected" or "operably linked" in the present context means placing a structural gene under the regulatory control of a promoter which then controls expression of the gene. Promoters and the like are generally positioned 5' (upstream) to the genes which they control. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the genetic sequence or promoter at a distance from the gene transcription start site that is approximately the same as the distance between that genetic sequence or promoter and the gene it controls in its natural setting, i.e. the gene from which the genetic sequence or promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e. the genes from which it is derived.

The *Agrobacterium*-mediated transformation method of the present invention preferably employs leaf bases and cores as the explant material. Following co-cultivation with *Agrobacterium* species, these materials form organogenic callus which are then used to regenerate plants.

Accordingly, in a particularly preferred embodiment, the present invention provides a method for producing a genetically modified monocotyledonous plant, said method comprising:

obtaining an explant from said plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without the *Agrobacterium* overgrowing the plant cells;

selecting for the transformed plant cells and permitting the cells to form organogenic callus; and

regenerating a plant from said transformed organogenic callus.

The preferred monocotyledonous plant of the present invention is a member of the Bromiliadeae family such as but not limited to pineapples and ornamental plants and members outside this family such as but not limited to ginger, sugar cane and cereal plants. Reference to particular plants includes reference to related species. A related species of pineapple includes other members of the Bromiliadeae, banana and ginger plants, cereal plants and any plant comprising plant cells transformable by the instant transformation method. The present invention also extends to parts of the genetically modified monocotyledonous plants and in particular parts of the genetically modified pineapple plants including cells, tissues, organs and fruit, reproductive parts and non-cellular material of genetically modified pineapple plants.

Accordingly, a particularly preferred embodiment of the present invention provides a method of transforming cells of a pineapple plant or a related plant with genetic material, said method comprising:-

obtaining explant from said pineapple plant or a related species;

co-cultivating same with *Agrobacterium* species having T-DNA or T-DNA region comprising genetic material to be transformed into said pineapple plant cells for a time and under conditions sufficient for transfer of the genetic material to occur; and

selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus.

In a related embodiment, the present invention contemplates a method of genetically modifying a pineapple or related plant, said method comprising:-

obtaining an explant from a pineapple or related plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transferred into the pineapple or related cells for a time and under conditions sufficient for the genetic material to transfer to said cells;

selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus; and

regenerating a pineapple or related plant from said organogenic callus.

Generally, after co-cultivating the pineapple or related cells with *Agrobacterium* species, the transformed cells are cultured to form organogenic callus and this is used to regenerate plants. In accordance with the present invention, the transformation/regeneration procedure does not employ embryogenic callus.

The *Agrobacterium* species may be either high or low virulent strain. A particularly useful *Agrobacterium* species is *Agrobacterium tumefaciens* strain AGL0 (Lazo *et al.*, 1991), AGL1 (Lazo *et al.*, 1991), strain ICMP 8317 (Janssen and Gardner, 1989), strain EHA101 (Hood *et al.*, 1986), strain LBA4404 (Hoekema *et al.*, 1983) and strain C58 (Konez and Schell, 1986). Strain AGL0 is particularly preferred.

Particularly useful genes or genetic material encode one or more of polyphenol oxidase, ACC synthase, ACC oxidase, malic enzyme, malic dehydrogenase, glucose oxidase, chitinase, defensin, expansin, hemicellulase, xyloglucan transglycosylase, an apetala gene, a leafy gene, a knott-related gene, a homeobox gene, a ribonuclease or a ribozyme amongst others.

Particularly useful traits to modulate in pineapple or related plants include but are not limited to herbicide resistance, pesticide resistance, disease resistance, environmental tolerance (e.g. salt, cold, heat, water, stress), morphology, growth characteristics, nutritional content, taste, sugar content, yield, horticultural characteristics, consumer quality traits amongst others.

The method of the present invention is initiated by obtaining an explant from the plant, generally in the form of leaf bases or cores obtained from *in vitro* grown shoot cultures. A suitable strain of *Agrobacterium*, such as but not limited to strain AGL0, is then cultured, washed and resuspended in a medium such as MS salts comprising from about 10 μM to about 500 μM acetosyringone or a functional homologue thereof and preferably about 100 μM acetosyringone or a functional homologue thereof. The explants are placed in a slurry of *Agrobacterium*. The *Agrobacterium* is then subjected to a vacuum such as but not limited to vacuum infiltration for 1-30 minutes, generally for 2-10 minutes and preferably for about 5 minutes. The explants are then generally co-cultivated with the *Agrobacterium* for from about one to about eight weeks, more particularly from about two to about six weeks and even more particularly from about three to about five weeks, such as four weeks, in MS salts of a plant hormone or combination of plant hormones, and one or more plant hormones such as cytokinins and auxins and gibberellins.

Suitable cytokinins include benzyl aminopurine (BAP), benzyl adenine (BA), thidiazuron (TDZ), kinetin, zeatin as well as the endosperm obtained from the seed of immature coconut seeds. Suitable auxins include napthalene acetic acid (NAA), indole acetic acid (IAA), indole butyric acid (IBA) and 2, 4-dichlorophenoxy-acetic acid (2, 4-D). Any

combination of auxin and gibberellin may be used and the concentrations of plant growth hormones used may vary from about 0.1 mg/l to about 50 mg/l, typically from about 1 mg/l to about 30 mg/l and preferably 10 mg/l. Examples of suitable gibberellins include A1, GA1, GA8, GA2, GA3, GA4, GA5, GA6, GA7 and GA8.

After co-cultivation, the explants are blotted dry. Transformed cells are then subjected to selection. For example, selection of transformed cells may be achieved using a variety of chemical agents, such as antibiotics (e.g. hygromycin and/or geneticin (G418)) or herbicides (e.g. BASTA (trademark)). Use of these agents may require the inclusion of a selectable marker gene, such as but not limited to the *hph* gene for hygromycin resistance or the *npt*II gene for geneticin or kanamycin resistance. These genes render the transformed cells resistant to the selection agent. One particularly useful selection medium consists of MS salts, supplemented with BAP, NAA, cefotaxime, and geneticin (G418) or hygromycin, although a range of other suitable media may be employed, for example, one which replaces geneticin or hygromycin with kanamycin and in particular high concentrations of kanamycin. Geneticin may be used, for example, in the range of 50 µg/l to 500 mg/l, typically 50 µg/l to 200 mg/l and preferably 100 µg/l to 50 mg/l. Similar concentrations of hygromycin may also be used. Under certain circumstances, kanamycin may also be used.

Transformed organogenic callus is apparent after about six weeks and selection may be continued for a further from about one to about six months and preferably from about three to four months. Reference herein to a "week" generally means seven days although in some cases, a week may be regarded as five week days. Shoot formation is then initiated by contacting the cells in appropriate medium such as MS salts, coconut water and a casein. Propagation of shoots generally requires a cytokine such as BAP. Production of roots generally requires an auxin. Accordingly, roots were induced in media supplemented with 1 mg/l NAA although roots may also be formed on hormone free media. The plants are then transferred to soil.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Explant

Explants for transformation were obtained from the base of leaves of *in vitro* grown shoots. Such shoot cultures were derived from surface sterilized axillary buds excised from crown shoots of field grown fruit. Following sterilisation, shoots were propagated on Shoot Multiplication Media (MSM Media; Hamill *et al.*, 1992), which consists of MS salts + 2.5 mg/l BAP. On this media, side shoots were produced, which were excised and either transferred to MSM or maintained on basal MS media. Plants used for transformation were maintained on basal MS medium.

Explants for co-cultivation consisted of approximately 0.5 cm sections of the base of individual leaves, the region of leaf used corresponded roughly to the zone where chlorophyll accumulation commences. Leaf colour in this region ranges between white and green.

EXAMPLE 2

Co-cultivation with Agrobacterium

Agrobacterium tumefaciens strain AGL0 was transformed with binary vectors containing constructs designed to express a number of genes as shown in Table 1. These constructs contained a selectable marker gene (*npt*II or *hph*), some also contained the GUS or GFP marker gene. *Agrobacterium* were plated on LB agar and grown for 2 to 3 days at 28°C. A liquid starter culture (typically 3 to 10 ml) was initiated from these in YEB media (see Example 8) and grown for 1 to 2 days at 28°C with shaking.

When the starter culture reached an OD 600 nm between 1.0 and 2.0, 1.5 ml (when OD was 1.0) or 100 Fl (when OD was 2.0 or more) of starter culture was seeded into a 100 ml YEB, which was incubated overnight at 28°C with shaking until the culture reached an OD 600 nm of 0.6.

All subsequent manipulations were performed on ice. *Agrobacterium* were washed twice prior to use for co-cultivation. Cells were harvested by centrifugation at 8,000 rpm (Beckman JA14 rotor) and resuspended in one half volume of liquid MS media supplemented with 100 FM acetosyringone. Cells were again harvested as above and resuspended in one third volume of MS supplemented with 100 FM acetosyringone. The OD 600 nm of these suspensions was measured and adjusted to 1.4 to 1.6 using MS supplemented with 100 FM acetosyringone. Cells were kept on ice until used.

For co-cultivation, pineapple leaves were removed from shoot cultures and suitable explants were cut from leaves in the bacterial suspension, which assisted in penetration of the leaf pieces by *Agrobacterium.* About 125 leaf pieces were treated with 15 to 20 ml of bacterial suspension in an appropriate tissue culture dish.

To further assist entry of *Agrobacterium,* freshly cut explants in bacterial suspension were subjected to a vacuum treatment by placing leaf pieces in the bacterial suspension in a DNA SpeedVac (DNA 100; Savant) and applying the vacuum, usually for 5 minutes. This is generally referred to as "vaccum infiltration". Explants were removed, blotted dry then placed on Callus Media (CAL) supplemented with 100 FM acetosyringone. CAL media consists of MS basal media supplemented with 10 mg/l BAP and 10 mg/l NAA.

Plates were co-cultivated in the dark for two days and co-cultivation continued in a growth chamber for at least 2, preferably 3 to 4 weeks. The growth chamber was maintained at 25°C; lighting was provided by fluorescent tubes with 16 hours light and 8 hours dark. Under these conditions outgrowth of *Agrobacterium,* which typically occurs during co-cultivation of other species after 2 to 3 days, was minimal. Initiation of organogenic callus was observed on some explants during the co-cultivation phase.

EXAMPLE 3

Selection and transformation of callus

Following co-cultivation, explants were transferred to CAL media supplemented with cefotaxime (300 mg/l), to kill *Agrobacterium,* and geneticin (100 mg/l) to select for growth of transformed pineapple cells. Explants were transferred every 3 to 4 weeks onto fresh CAL media supplemented with cefotaxime (300 mg/l) and geneticin (100 mg/l). After 3 to 5 months, rapidly-growing, green, granular callus was observed on some explants. Such geneticin-resistant, putatively transformed, callus was excised from the explant and transferred to CAL media supplemented with geneticin (100 mg/l) for maintenance and expansion.

EXAMPLE 4

Shoot initiation from transformed cells

For shoot initiation, callus pieces were transferred to Plant Regeneration media (PRR; Rangan, 1982), which consisted of MS media supplemented with 50 g/l sterile coconut water (Sigma) as a cytokinin source and 400 mg/l casein hydrolysate. Under these conditions, shoots appeared rapidly and could be excised from the callus after 4 to 8 weeks culture, then transferred to MS media for elongation and further development.

EXAMPLE 5

Root initiation and transplantation to soil

Once *in vitro* grown shoots reached about 3-4 cm, roots were initiated by auxin treatment in MS salts supplemented with 1 mg/l NAA, although roots also initiated well on basal MS media. Root initiation was apparent after 4 to 6 weeks and plantlets were ready for transfer to soil in a glasshouse at this time.

Planting in soil in the glasshouse required an extended acclimatisation procedure. Plantlets were planted in 4" pots in water-saturated potting mix (e.g. 1 peat:2 sand) in a humidifying cabinet under shade cloth. Humidity was slowly decreased to ambient levels over a one month period and light intensity was gradually increased over a two to three month period by progressively removing shade cloth. Plants were then transferred to 6" pots and grown in the glasshouse. For planting in the field, plants were first acclimatized in shade houses for two to four months before planting.

EXAMPLE 6

Characterization of transgenic plants

1. Preparation of gene constructs

Pineapple was transformed with binary constructs designed to express the *npt*II selectable marker gene. In some instances these constructs also expressed a second gene, either the GUS or GFP marker gene as outlined in Table 1. A generalized map of these constructs is shown in Figure 1.

TABLE 1 Binary constructs used to transform pineapple

Construct designation	Selectable marker construct	Second gene construct	Reference for derivation
pBIN.mGFP5-ER	nos:*npt*II[1]	35S:GFP[3,4]	Haseloff *et al.* (1995)
pART27.GUS	nos:*npt*II	35S:GUS[5]	Gleave *et al.* (1993)
pBS427	Sc4:*npt*II[2]	none	Boevink *et al.* (1995)
pBS427.GUS	Sc4:*npt*II	35S:GUS	This specification

1 nos:*npt*II means that the nos promoter is operably linked to the *npt*II gene

2 Sc4:*npt*II means that the Sc4 promoter is operably linked to the *npt*II gene

3 35S means cauliflower mosaic virus (CaMV) 35S promoter

4 35S:GFP means that the 35S promoter is operably linked to the GFP gene

35S:GUS means that the 35S promoter is operably linked to the GUS gene

The plasmid, pBIN.mGFP5-ER, is described by Haseloff *et al.* (1995) and comprises the CaMV 35S promoter operably linked to the GFP gene.

The construct pART27.GUS was designed to express the GUS marker gene under control of the CaMV 35S promoter. It was made by cloning the GUS coding sequences from pBKS.GUS (Farrell and Beachy, 1990) as a *Sal*I - *Sma*I fragment into *Xho*I - *Sma*I cut pART7 (Gleave *et al.*, 1992). The resulting plasmid, pART7.GUS, was cloned into pART27 as a *Not*I fragment to create pART27.GUS.

The plasmid pBS427 is a derivative of pART27 (Gleave *et al.*, 1992). In pBS427, the selectable marker cassette (nos-*npt*II-nos from pART27) is replaced by an alternative marker cassette. In pBS427, expression of the *npt*II gene is controlled by the Sc4 promoter and Sc5 terminator (Boevink *et al.*, 1995). In addition, the *npt*II gene is interrupted by an intron from castor bean catalase gene (Wang *et al.*, 1997). The modified selection cassette is, therefore, Sc4-*npt*II (intron)-Sc5.

The plasmid pBS427.GUS was created by cloning pART7.GUS as a *Not*I restriction fragment into *Not*I pBS427 digested.

Binary constructs were assembled in *E. coli* using standard techniques, then transformed into *Agrobacterium tumefaciens* strain AGL0 using tri-parental mating.

Following co-cultivation of pineapple leaves with *Agrobacterium*, putative transgenic callus appeared within two to four months of culture. This callus grew rapidly on geneticin containing CAL plates, whereas non-transformed callus grew poorly, if at all, on this media, before explants darkened and died. To confirm callus was transformed, GFP or GUS assays were performed.

2. Histochemical assays

For GUS assays, callus or regenerated leaf tissues were incubated in GUS staining solution consisting of 100 mM sodium phosphate (pH 7.0), 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl-β-D glucuronic acid), 4.5 mM potassium ferricyanide, 4.5 mM potassium ferrocyanide, 0.25% (v/v) Triton X-100, 10% (v/v) methanol, 4% (v/v) dimethyl sulphoxide and incubated for 16 to 48 hrs at 37°C. Samples were usually destained at 37°C in 70% v/v ethanol for 16 to 48 hrs. GUS-positive tissues stained blue after this treatment. An example of pART27.GUS-transformed pineapple is shown in Figure 2.

Visualization of GFP fluorescence in plant cells (callus and regenerated tissues) was performed as described by Elliott *et al* (1999). Fluorescence was observed using a Leica MZ6 stereomicroscope with fluorescence GFP plus filter module (Leica Microscopy and Scientific Instruments, Switzerland) which contains a 480/40 nm excitation filter, 505 nm LP dichromatic beam-splitting mirror and 510 nm LP barrier filter. A 50 W high-pressure mercury vapour lamp provided illumination. A narrow bandpass interference filter (S550/50 NP) was introduced to minimize red chlorophyll autofluorescence, when required. Under these conditions, transformed tissue gave bright green fluorescence. An example of pBIN.mGFP5-ER-transformed callus is shown in Figure 3.

3. PCR assay

To confirm marker gene assays, DNA samples, isolated from callus or transgenic shoots using the method of Thomson and Henry (1995), were assayed by PCR. Approximately 100 mg of tissue was placed in a microfuge tube containing 20 µl of 100 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0), 1 M KCl and ground with a small pestle. The samples were incubated for 10 min at 95°C. One microlitre of a 1:10 dilution of this was used as a PCR substrate.

PCR reactions were undertaken in 25 μl reactions containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM (or 4 mM) $MgCl_2$, 200 μM dNTPs, 0.2 μM primers, 0.5 U *Taq* polymerase (Boehringer Mannheim) using a Corbett PC-960G gradient thermal cycler. Reaction conditions involved an initial denaturation step at 94°C for 90 sec, followed by 30 cycles of 94°C for 20 sec, 55°C for 30 sec and 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. PCR products were analyzed by electrophoresis on 1% (w/v) agarose gels.

Oligonucleotide primers used in these analyses were specific for the *npt*II, GUS or GFP genes. These sequences were:

"*npt*II":

KAN-1: 5'-GGA TCC ATG ATT GAA CAA GAT GGA TTG CAC-3' <400>1

KAN-2: 5'-TCT AGA TCA GAA GAA CTC GTC AAG AAG GCG-3' <400>2

"gfp-ER":

m-gfp 5': 5'-GGA TCC AAG GAG ATA TAA CAA TGA AG-3' <400>3

ER-2: 5'-TAA CTC GAG TTG TAT AGT TCA TCC ATG CC-3' <400>4

"gus":

GUS-1: 5'-CGA TAC CTC TCT TTA GGC ATT GG-3' <400>5

GUS-2: 5'-TCA TTG TTT GCC TCC CTG CTG CG-3' <400>6

For the *npt*II gene, a fragment of approximately 800 bp was amplified with the *nptII* primers from tissues transformed with the pBIN.mGFP-5ER or pART27.GUS binary vectors. For tissues transformed with the pBS427 or pBS427.GUS binary vector, an approximately 1,000 bp fragment was amplified with the *npt*II primers; the latter constructs contain an intron within the *npt*II gene which lies between the primer sites. For tissues transformed with pBIN.mGFP-5ER, an approximately 820 bp fragment was generated with the gfp-ER primers (<400>3 and <400>4). For pART27.GUS and pBS427.GUS transformed tissues, an approximately 750 bp fragment was amplified with the gus primers (<400>5 and <400>6). An example of PCR reactions is shown in Figure 4.

A summary of histochemical and PCR assay results obtained in transformation experiments is listed in Table 2.

TABLE 2 Summary of pineapple transformation experiments showing frequency of transformation and status of assays used to confirm that tissues were transformed (nd - not done)

Binary construct	Number of transformed callus/number of leaf explants (% transformation)	GUS assays	GFP assays	PCR assays
pBIN.mGFP5-ER	6/283 (2.1%)	nd	Callus all + ve	All + ve for *npt*II and GFP
pART27.GUS	3/200 (1.5%)	Callus all + ve	nd	All + ve for *npt*II and GUS
pBS427	35/1004 (3.5%)	nd	nd	15/15 callus pieces +ve for *npt*II
pBS427.GUS	4/200 (2.0%)	Callus all + ve	nd	All +ve for *npt*II and GUS

4. Southern Hybridization

Southern blot analysis is used for final confirmation of transformation. Genomic DNAs were prepared from transgenic shoots using the technique of Graham *et al* (1994). Approximately 10 µg of DNA was cleaved with a suitable restriction enzyme (e.g. *Bam*HI, *Hind*III), separated on an agarose gel (1% w/v) by electrophoresis and transferred to nylon Hybond N plus filters (Amersham) using capillary transfer with 20 x SSC (Standard saline citrate; 20 x SSC is 3M NaCl, 0.3 M Tri-sodium citrate; pH 7.0) according to the manufacturer's instructions. Following transfer, DNA was cross-linked to the filter using a UV chamber (Bio-Rad).

Probes were prepared by ^{32}P-labelling DNA fragments of the GUS, GFP and *npt*II genes generated from PCR reactions (described above) using a random priming kit according to the manufacturer's (Amersham) directions.

Filters were pre-hybridized in a solution of 2 x SSC, 5 x Denhardt's solution (5 x Denhardt's is 0.5 g/l Ficoll, 0.5 g/l polyvinylpyrrolidine, 0.5 g/l bovine serum albumin), 0.1% (w/v) SDS and 100 µg/ml denatured single stranded DNA at 65°C for 2 hrs. Probes were denatured by boiling, added to fresh hybridization buffer described above and incubated at 65°C for 16 hours. To remove unhybridized probe, filters were washed three times in 2 x SSC, 0.1% (w/v) SDS at 65°C for 30 min each wash, then exposed to X-ray film.

The ideal choice of restriction enzyme and probe varied for tissue transformed with different constructs. For DNA from plants transformed with pART27.GUS, for example, transgene copy number can be best estimated by restricting genomic DNA with *Hind*III and probing with a fragment derived from the *ocs* terminator region of the pART7 vector (Gleave *et al.*, 1993).

Results for two control, non-transgenic plant lines and five putative transgenic lines are set forth in Table 3 below:-

TABLE 3

#	Line	Gene transformed with	Gene probed for	Result
1	control	non-transformed	GUS	-ve
2	51-1	GFP	GUS	-ve
3	68-7	GUS	GUS	+ve
4	68-6.2	GUS	GUS	+ve
5	control	non-transformed	GFP	-ve
6	V48	*npt*II only	GFP	-ve
7	66-3.5	GFP	GFP	+ve

As additional internal negative controls, plants transformed with GFP and *npt*II were also probed for GUS and GFP, respectively and found to be negative (#2 and #6, above). Lines 68-7 and 68-6.2 were found to have multiple bands indicating the presence of integrated GUS gene in the plants genome. Likewise, line 66-3.5 displayed multiple bands indicating successful tranformation with the GFP gene-containing construct.

EXAMPLE 7

Somaclonal variation

A transformation regeneration system should result in minimal somaclonal variation. To ascertain likely difficulties with this system, various control experiments were undertaken. A large number of plants were regenerated *in vitro* without transformation, transplanted to soil, allowed to mature, then planted in the field. Of 102 plants, only 6.4% showed spiny phenotypes and 2.4% showed dwarf growth habit. These phenotypes are crude indicators of somaclonal variation and provide evidence that normal plants can be obtained from this regeneration system, at high frequency. In addition, plants from 24 transformation events

with pBS427, were transplanted into soil in a glass house. For most lines, two or three clonal replicates were planted. From a total of 46 plants, only one showed evidence of somaclonal variation. Taken together, these data indicate that the level of somaclonal variation with the transformation system of the present invention is quite low.

EXAMPLE 8

Media

The following media were used in the Examples:-

1. Media for growth of Agrobacterium

LB agar

Agrobacterium cultures were maintained on LB agar plates supplemented with rifampicin as follows;

Bacto-tryptone	10 g/l
Bacto-yeast extract	5 g/l
NaCl	10 g/l
Bacto-agar	15 g/l
Rifampicin	30 mg/l

For *Agrobacterium* transformed with pBIN.mGFP5-ER, LB agar was further supplemented with:

Kanamycin	50 mg/l

For *Agrobacterium* transformed with pART27.GUS, pBS427 or pBS427.GUS, LB agar was further supplemented with:

Spectinomycin	100 mg/l

YEB liquid media

For growth of liquid cultures of *Agrobacterium*, YEB media supplemented with rifampicin was used:

Bacto-yeast extract	1 g/l
Bacto-beef extract	5 g/l
Bacto-peptone	5 g/l
$MgSO_4.7\ H_2O$	0.5 g/l
Sucrose	5 g/l
Rifampicin	30 mg/l

For *Agrobacterium* transformed with pBIN.mGFP5-ER, this YEB media was further supplemented with:

Kanamycin	50 mg/l

For *Agrobacterium* transformed with pART27.GUS, pBS427 or SCSV4.GUS, YEB media was further supplemented with:

Spectinomycin	100 mg/l

2. *Media for growth and maintenance of pineapple shoots*

For plant tissue culture, all media were based on Murashige Minimal Salts Media (MS). Commercial preparations from Gibco BRL were used, which consisted of:

$(NH_4)NO_3$	1,650 mg/l
H_3BO_3	6.2 mg/l
$CaCl_2$	332.2 mg/l
$CoCl_2$	0.014 mg/l
$CuSo_4$	0.016 mg/l
Na_2EDTA	37.25 mg/l
$FeSO_4.7H_2O$	27.8 mg/l
$MgSO_4$	180.70 mg/l
$MnSO_4.H_2O$	16.9 mg/l
$Na_2MoO_4.2H_2O$	0.25 mg/l
KI	0.83 mg/l
KNO_3	1,900 mg/l
KH_2PO_4	170 mg/l
$ZnSO_4.H_2O$	5.37 mg/l
i-Inosotol	100 mg/l
Thiamine.HCl	0.4 mg/l
Sucrose	30 g/l

This media were further supplemented with vitamins (Sigma) to a final concentration of:

Glycine	2 mg/l
Myo-inosotol	100 mg/l
Nicotinic acid	0.5 mg/l
Pyridoxine.HCl	0.5 mg/l
Thiamine.HCl	0.1 mg/l

All pineapple tissue culture was performed on solid agar, using tissue culture plates or tubs. To prepare solid medium, 0.3% w/v Phytagel (Sigma) was added to basal MS medium supplemented with vitamins.

MS Plates

Pineapple plants for transformation were maintained on solid basal MS media supplemented with vitamins as described above. Rooted transgenic shoots were also maintained on the same media.

Shoot Multiplication (MSM) plates

For multiplying pineapple shoots of both non-transformed and transgenic pineapple, plants were cultured in solid MS media supplemented with vitamins, further supplemented with:

BAP	2.5 mg/l

3. Media for pineapple transformation

Liquid MS media

For washing *Agrobacterium* and co-cultivation with pineapple leaf pieces, liquid MS medium supplemented with vitamins was used. This was further supplemented with:

Acetosyringone	100 µM

Callus (CAL) Plates

For callus induction, explants were cultured on MS agar supplemented with cytokinin (BAP) and auxin (NAA) to induce callus formation. For co-cultivation, MS plates supplemented with vitamins were used. This medium was further supplemented with:

BAP	10 mg/l
NAA	10 mg/l

To select for transformed tissues, CAL media was further supplemented with geneticin, to select for transformed cells, and cefotaxime to kill *Agrobacterium,* as follows:

Geneticin	100 mg/l
Cefotaxime	300 mg/l

For maintenance and expansion of transformed callus, CAL plates were supplemented with only geneticin to maintain selection of transformed tissues, as follows:

Geneticin	100 mg/l

Plant Regeneration (PRR) Plates

For shoot induction from both transgenic and non-transformed callus, callus pieces were transferred to PRR plates which consisted of solid basal MS media, supplemented with vitamins, further supplemented with:

Sterile coconut water (Sigma)	50 g/l
Casein hydrolysate	400 mg/l

Root Promoting (RP) Plates

To induce roots on transgenic shoots, shoots of about 3-4 cm in length were excised and transferred to RP plates which consisted of solid basal MS media supplemented with vitamins, further supplemented with:

NAA	1 mg/l

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS

1. A method of transforming cells of a monocotyledonous plant with genetic material, said method comprising:-

obtaining an explant from said plant;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without said *Agrobacterium* overgrowing the plant cells; and

selecting for the transformed plant cells and permitting the cells to form organogenic callus.

2. A method according to Claim 1 wherein the plant is a pineapple plant or related species or parts thereof.

3. A method according to Claim 2 wherein the parts of the pineapple plant include tissues, organs and/or reproductive parts.

4. A method according to Claim 1 or 2 or 3 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* species to a vaccum.

5. A method according to Claim 4 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* to vacuum infiltration.

6. A method according to any one of Claims 1 to 5 wherein the co-cultivation is for from about one to about eight weeks.

7. A method according to Claim 6 wherein the co-cultivation is for from about two to about six weeks.

8. A method according to Claim 7 wherein the co-cultivation is for from about three to about five weeks.

9. A method according to any one of Claims 1 to 8 wherein the *Agrobacterium* species used in the co-cultivation is washed in a medium supplemented with a virulence inducer.

10. A method according to Claim 9 wherein the virulence inducer is acetosyringone.

11. A method according to any one of Claims 1 to 10 wherein the genetic material comprises a coding sequence for a mRNA molecule.

12. A method according to Claim 11 wherein the mRNA molecule is translated to a peptide, polypeptide or protein.

13. A method according to any one of Claims 1 to 10 wherein the genetic material induces a mutation in a target sequence.

14. A method according to Claim 11 wherein the genetic material comprises a coding sequence operably linked to a promoter.

15. A method according to Claim 14 wherein the genetic material further comprises a terminator operably linked to the 3' end of the coding region.

16. A method according to Claim 14 wherein the promoter is recognized by a type I, II and/or III RNA polymerase.

17. A method according to Claim 16 wherein the promoter is recognized by a type I RNA polymerase.

18. A method according to Claim 16 wherein the promoter is recognized by a type II RNA polymerase.

19. A method according to Claim 16 wherein the promoter is recognized by a type III RNA polymerase.

20. A method according to any one of Claims 1 to 10 wherein the explant of the plant is a leaf base.

21. A method for producing a genetically modified monocotyledonous plant, said method comprising:-

obtaining explant from a plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transformed into said plant cells for a time and under conditions sufficient for the genetic material to transfer to plant cells without said *Agrobacterium* overgrowing the plant cells;

selecting transformed plant cells and permitting the cells to form organogenic callus; and

then regenerating a plant from selected transformed plant cells.

22. A method according to Claim 21 wherein the plant is a pineapple plant or related species or parts thereof.

23. A method according to Claim 22 wherein the parts of the pineapple plant include tissues, organs and/or reproductive parts.

24. A method according to Claim 21 or 22 or 23 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* species to a vaccum.

25. A method according to Claim 24 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* to vacuum infiltration.

26. A method according to any one of Claims 21 to 25 wherein the co-cultivation is for from about one to about eight weeks.

27. A method according to Claim 26 wherein the co-cultivation is for from about two to about six weeks.

28. A method according to Claim 27 wherein the co-cultivation is for from about three to about five weeks.

29. A method according to any one of Claims 21 to 28 wherein the *Agrobacterium* species used in the co-cultivation is washed in a medium supplemented with a virulence inducer.

30. A method according to any one of Claims 21 to 30 wherein the virulence inducer is acetosyringone.

31. A method according to Claim 22 wherein the genetic material comprises a coding sequence for a mRNA molecule.

32. A method according to Claim 31 wherein the mRNA molecule is translated to a peptide, polypeptide or protein.

33. A method according to any one of Claims 21 to 30 wherein the genetic material induces a mutation in a target sequence.

34. A method according to Claim 31 wherein the genetic material comprises a coding sequence operably linked to a promoter.

35. A method according to Claim 34 wherein the genetic material further comprises a terminator operably linked to the 3' end of the coding region.

36. A method according to Claim 34 wherein the promoter is recognized by a type I, II and/or III RNA polymerase.

37. A method according to Claim 36 wherein the promoter is recognized by a type I RNA polymerase.

38. A method according to Claim 36 wherein the promoter is recognized by a type II RNA polymerase.

39. A method according to Claim 36 wherein the promoter is recognized by a type III RNA polymerase.

40. A method according to any one of Claims 21 to 30 wherein the explant of the plant is a leaf base.

41. A method for producing a genetically modified monocotyledonous plant, said method comprising:

obtaining an explant from said plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a

time and under conditions sufficient for the genetic material to transfer into the plant cells without the *Agrobacterium* overgrowing the plant cells;

selecting for the transformed plant cells and permitting the cells to form organogenic callus; and

regenerating a plant from said transformed organogenic callus.

42. A method according to Claim 41 wherein the plant is a pineapple plant or related species or parts thereof.

43. A method according to Claim 42 wherein the parts of the pineapple plant include tissues, organs and/or reproductive parts.

44. A method according to Claim 40 or 41 or 42 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* species to a vaccum.

45. A method according to Claim 44 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* to vacuum infiltration.

46. A method according to any one of Claims 40 to 45 wherein the co-cultivation is for from about one to about eight weeks.

47. A method according to Claim 46 wherein the co-cultivation is for from about two to six weeks.

48. A method according to Claim 47 wherein the co-cultivation is for from three to five weeks.

49. A method according to any one of Claims 40 to 48 wherein the *Agrobacterium* species used in the co-cultivation is washed in a medium supplemented with a virulence inducer.

50. A method according to Claim 49 wherein the virulence inducer is acetosyringone.

51. A method according to any one of Claims 40 to 50 wherein the genetic material comprises a coding sequence for a mRNA molecule.

52. A method according to Claim 51 wherein the mRNA molecule is translated to a peptide, polypeptide or protein.

53. A method according to any one of Claims 40 to 50 wherein the genetic material induces a mutation in a target sequence.

54. A method according to Claim 51 wherein the genetic material comprises a coding sequence operably linked to a promoter.

55. A method according to Claim 54 wherein the genetic material further comprises a terminator operably linked to the 3' end of the coding region.

56. A method according to Claim 54 wherein the promoter is recognized by a type I, II and/or III RNA polymerase.

57. A method according to Claim 56 wherein the promoter is recognized by a type I RNA polymerase.

58. A method according to Claim 56 wherein the promoter is recognized by a type II RNA polymerase.

59. A method according to Claim 56 wherein the promoter is recognized by a type III RNA polymerase.

60. A method of transforming cells of a pineapple plant or a related plant with genetic material, said method comprising:-

obtaining explant from said pineapple plant or a related species;

co-cultivating same with *Agrobacterium* species having T-DNA or T-DNA region comprising genetic material to be transformed into said pineapple plant cells for a time and under conditions sufficient for transfer of the genetic material to occur;

selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus.

61. A method according to Claim 60 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* species to a vaccum.

62. A method according to Claim 61 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* to vacuum infiltration.

63. A method according to Claim 60 or 61 or 62 wherein the co-cultivation is for from about one to about eight weeks.

64. A method according to Claim 63 wherein the co-cultivation is for from about two to about six weeks.

65. A method according to Claim 64 wherein the co-cultivation is for from about three to about five weeks.

66. A method according to any one of Claims 60 to 65 wherein the *Agrobacterium* species used in the co-cultivation is washed in a medium supplemented with a virulence inducer.

67. A method according to Claim 66 wherein the virulence inducer is acetosyringone.

68. A method according to any one of Claims 60 to 67 wherein the genetic material comprises a coding sequence for a mRNA molecule.

69. A method according to Claim 68 wherein the mRNA molecule is translated to a peptide, polypeptide or protein.

70. A method according to any one of Claims 60 to 67 wherein the genetic material induces a mutation in a target sequence.

71. A method according to Claim 68 wherein the genetic material comprises a coding sequence operably linked to a promoter.

72. A method according to Claim 71 wherein the genetic material further comprises a terminator operably linked to the 3' end of the coding region.

73. A method according to Claim 71 wherein the promoter is recognized by a type I, II and/or III RNA polymerase.

74. A method according to Claim 73 wherein the promoter is recognized by a type I RNA polymerase.

75. A method according to Claim 73 wherein the promoter is recognized by a type II RNA polymerase.

76. A method according to Claim 73 wherein the promoter is recognized by a type III RNA polymerase.

77. A method of genetically modifying a pineapple or related plant, said method comprising:-

obtaining an explant from a pineapple or related plant to be genetically modified;

co-cultivating the explant with *Agrobacterium* species having a T-DNA comprising genetic material to be transferred into the pineapple or related cells for a time and under conditions sufficient for the genetic material to transfer to said cells;

selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus; and

regenerating a pineapple or related plant from said selected transformed cells.

78. A method according to Claim 77 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* species to a vaccum.

79. A method according to Claim 77 or 78 wherein the co-cultivation comprises subjecting the explant and *Agrobacterium* to vacuum infiltration.

80. A method according to Claim 77 or 78 or 79 wherein the co-cultivation is for from about one to about eight weeks.

81. A method according to Claim 80 wherein the co-cultivation is for from about two to about six weeks.

82. A method according to Claim 81 wherein the co-cultivation is for from about three to about five weeks.

83. A method according to any one of Claims 77 to 82 wherein the *Agrobacterium* species used in the co-cultivation is washed in a medium supplemented with a virulence inducer.

84. A method according to Claim 83 wherein the virulence inducer is acetosyringone.

85. A method according to any one of Claims 77 to 84 wherein the genetic material comprises a coding sequence for a mRNA molecule.

86. A method according to Claim 85 wherein the mRNA molecule is translated to a peptide, polypeptide or protein.

87. A method according to any one of Claims 77 to 84 wherein the genetic material induces a mutation in a target sequence.

88. A method according to Claim 85 wherein the genetic material comprises a coding sequence operably linked to a promoter.

89. A method according to Claim 88 wherein the genetic material further comprises a terminator operably linked to the 3' end of the coding region.

90. A method according to Claim 88 wherein the promoter is recognized by a type I, II and/or III RNA polymerase.

91. A method according to Claim 90 wherein the promoter is recognized by a type I RNA polymerase.

92. A method according to Claim 90 wherein the promoter is recognized by a type II RNA polymerase.

93. A method according to Claim 90 wherein the promoter is recognized by a type III RNA polymerase.

94. A method according to any one of Claims 1, 21, 41, 60 or 77 wherein the species of *Agrobacterium* is *Agrobacterium tumefaciens*.

95. A method according to Claim 94 wherein the strain of *A. tumefaciens* is AGL0, ICMP8317, EHA101, LBA4404 or C58.

96. A method according to Claim 95 wherein the *A. tumefaciens* strain is AGL0.

97. A genetically modified plant prepared by the method of any one of Claims 1, 21, 41, 60 or 77.

98. A genetically modified plant according to Claim 97 wherein the plant exhibits a modified phenotype selected from herbicide resistance, pesticide resistance, disease resistance, environmental tolerance, morphology, growth characteristics, nutritional content, taste, sugar content, yield, horticultural characteristics or consumer quality traits.

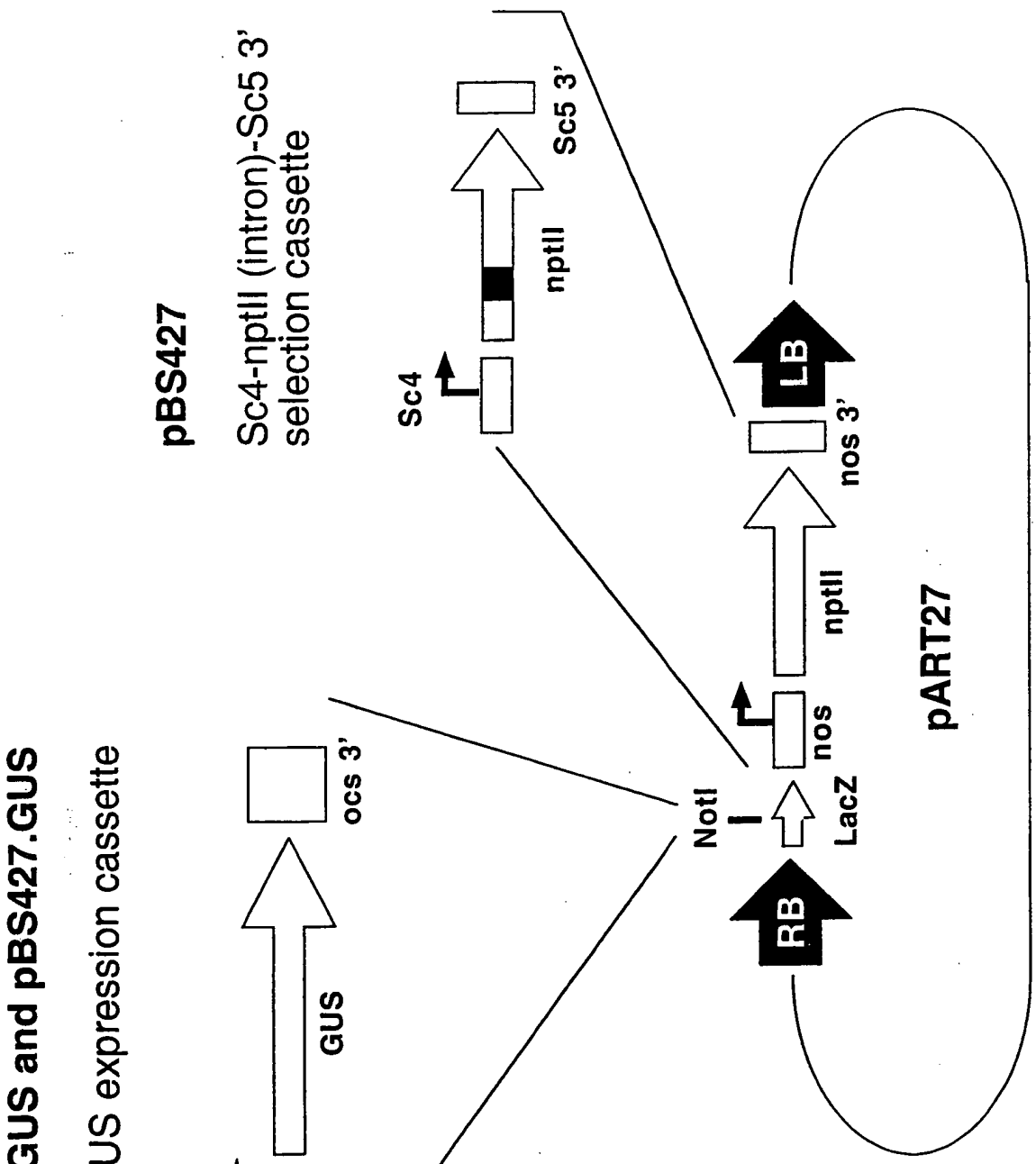
99. A genetically modified plant according to Claim 98 wherein the plant is a pineapple or related species.

100. A reproductive portion or part of a genetically modified plant according to Claim 97 or 98 or 99.

101. A piece of fruit harvested from a genetically modified plant according to Claim 97 or 98 or 99.

102. Use of a method of transformation of a plant cell comprising the steps set forth in any one of Claims 1, 21, 41, 60 or 77 in the manufacture of a genetically modified plant.

103. Use according to Claim 102 wherein the plant is a pineapple plant or related species.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pART7.GUS expression cassette
35S
GUS
ocs 3'
pBS427
Sc4-nptII (intron)-Sc5 3'
selection cassette
Sc4
nptII
Sc5 3'
NotI
LacZ
nos
nptII
nos 3'
RB
LB
pART27

Figure 1

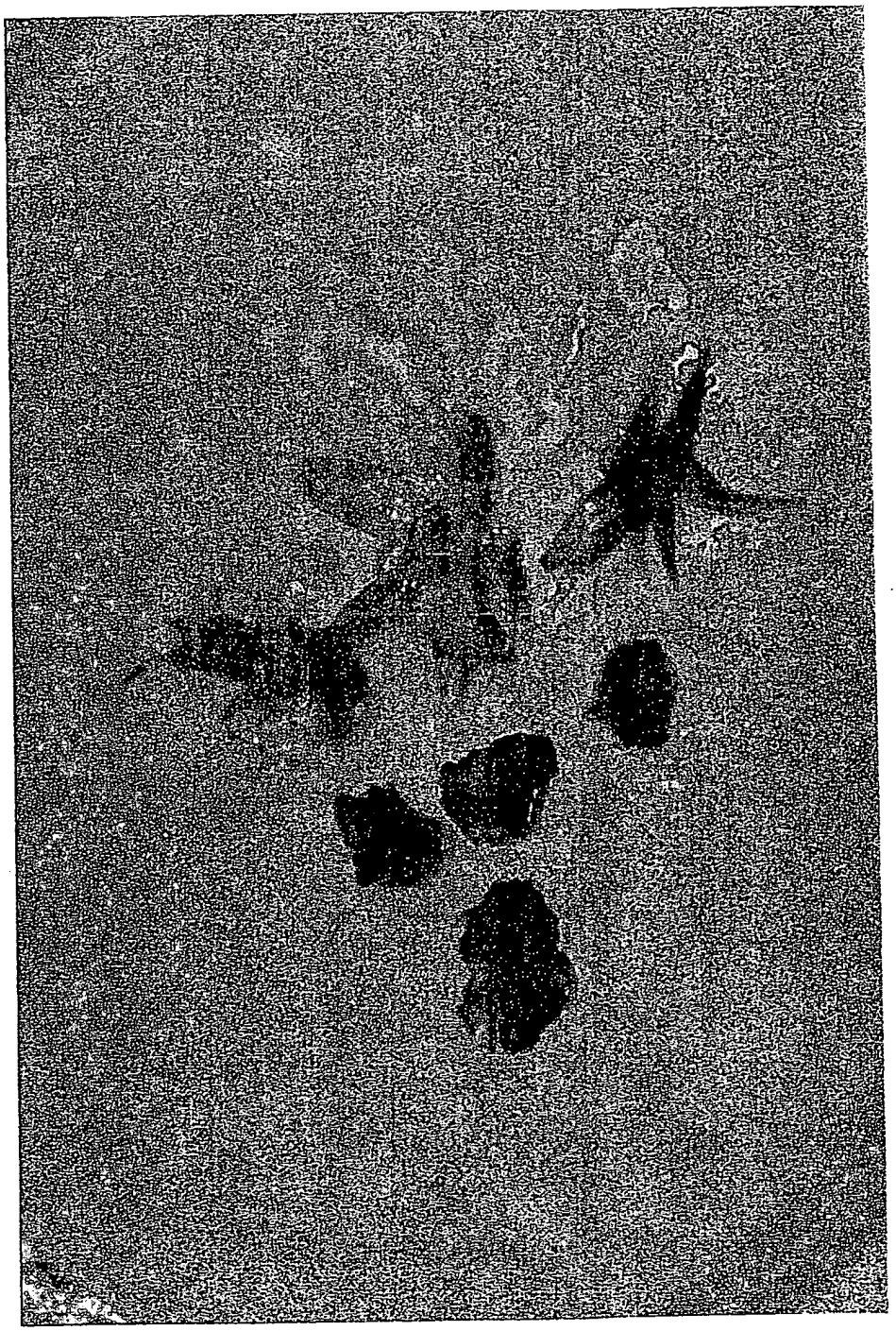

Figure 2

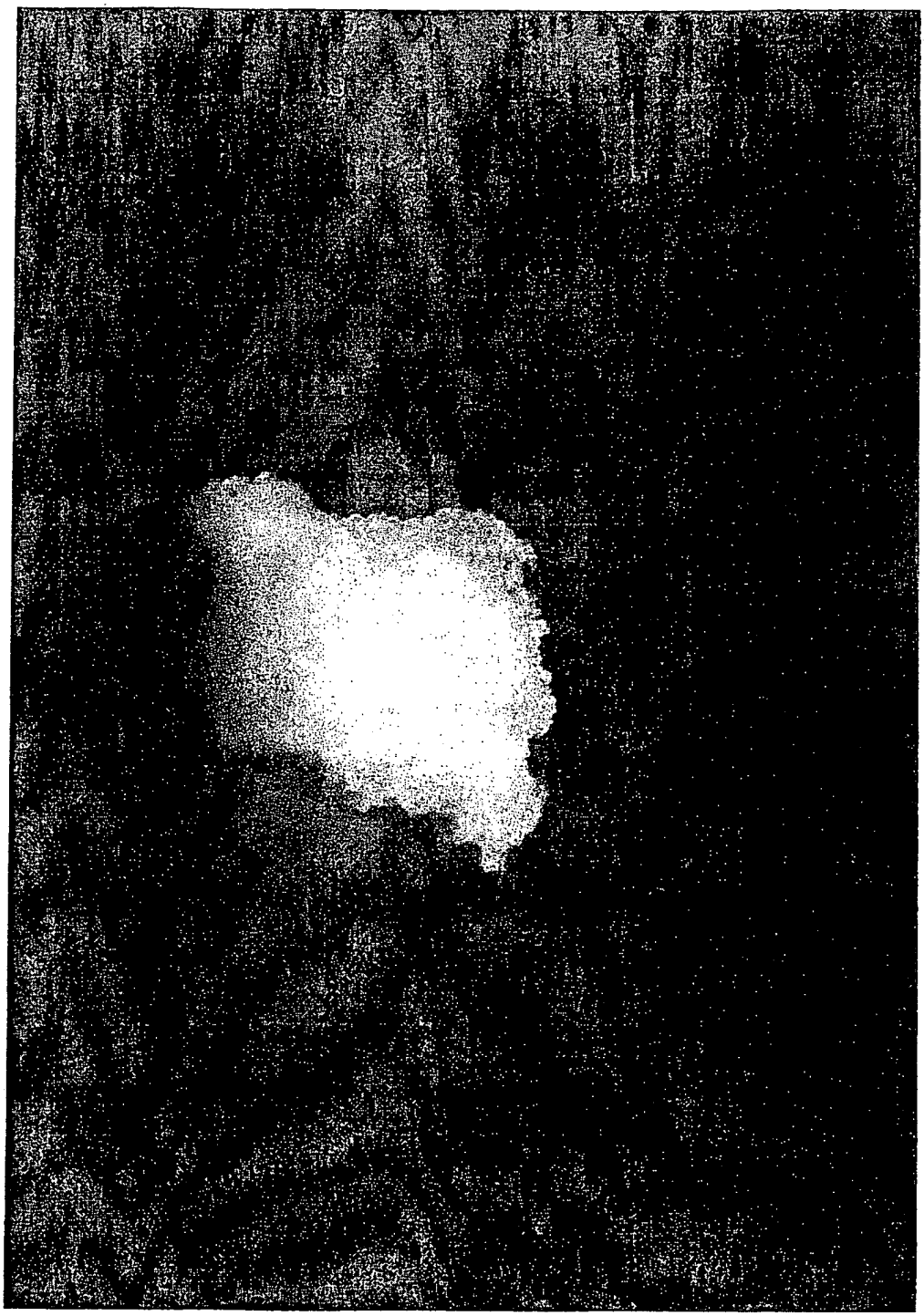

Figure 3

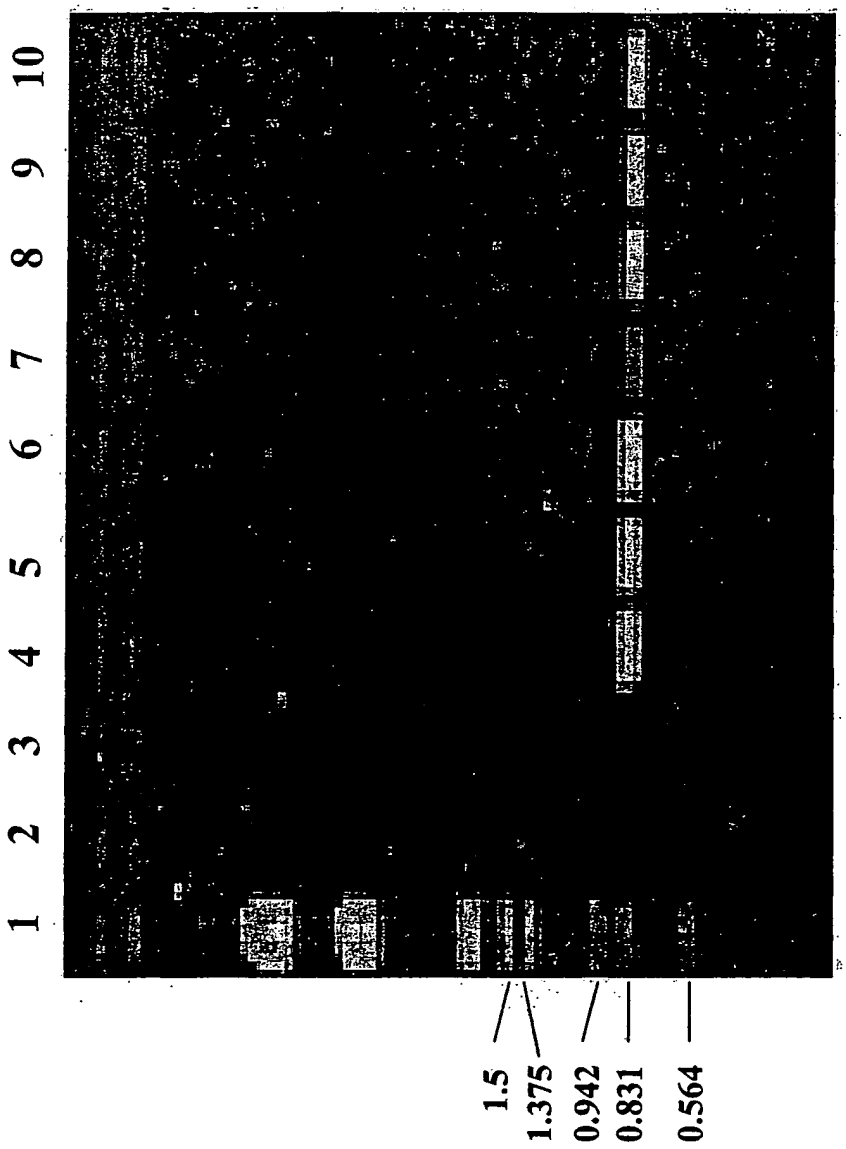

Figure 4

SEQUENCE LISTING

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<120> A METHOD OF TRANSFORMATION

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<140> International
<141> 2000-11-03

<150> PQ3883/99
<151> 1999-11-05

<160> 6

<170> PatentIn Ver. 2.1

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<213> synthetic construct

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01350

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A01H 1/00, 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

DERWENT, CHEMICAL ABSTRACTS, AGRICOLA, MEDLINE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS, CA, MEDLINE, AGRICOLA and keywords: (pineapple OR monocot?) AND [(agrobacter?) AND (transform? OR transgen?)].

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WANG et al. Improved vectors for *Agrobacterium Tumefaciens*-Mediated Transformation of Monocot Plants. Acta Horticulturae. August 1998, Vol. 461, pages 401-407.	
X	Refer to abstract, page 402 para.2 and page 403 para.2.	1,4-19,41,44-59 97,98,100,102
Y		20, 40
	TINGAY et al. *Agrobacterium Tumefaciens*-Mediated Barley Transformation. The Plant Journal. June 1997, Vol. 11(6), pages 1369-76	
X	Refer to page 1370, Results and pages1374-75, Experimental Procedures.	1,4-19,41,44-59 97,98,100,102
Y		20, 40
	WO 99/19499 A (OMS INVESTMENTS) 22 April 1999	
Y	Refer to page 8 line 29 to page 9 line 7 and fig 2.	20, 40

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 2000	-8 JAN 2001
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	-8 JAN 2001 ANDREW ACHILLEOS Telephone No : (02) 6283 2280

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01350

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARK et al. T-DNA Integration into Genomic DNA of Rice Following *Agrobacterium* Inoculation of Isolated Shoot Apices. Plant Molecular Biology. December 1996, Vol. 32(6). pages 1135-48. Refer to abstract and page 1137, Cocultivation of Agrobacteria and culture conditions.	41,44-59 97,98,100 102

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/01350

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	99/19499	AU	98066/98	US	5948956

END OF ANNEX